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Phosphorylation of the RAP74 Subunit of TFIIF Correlates with Tat-Activated Transcription of the HIV-1 Long Terminal Repeat

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Transcription from the HIV-1 long terminal repeat (LTR) is regulated by the viral transactivator Tat, which increases RNA polymerase II (RNAP II) processivity. Previous reports have demonstrated that phosphorylation of the RNAP II carboxy-terminal domain by TFIIH and P-TEFb is important for Tat transactivation. Our present results demonstrate that phosphorylation of the RAP74 subunit of TFIIF is also an important step in Tat transactivation. Interestingly, while the general transcription factor TFIIF is required for both basal and Tat-activated transcription, phosphorylation of the RAP74 subunit occurs in the presence of Tat and correlates with a high level of transcription activity. Using a biotinylated DNA template transcription assay, we provide evidence that RAP74 is phosphorylated by $TAF_{II}250$ during Tat-activated transcription. Depletion of RAP74 from the HeLa nuclear extract inhibited HIV-1 LTR-driven basal transcription and Tat transactivation. The addition of TFIIF, reconstituted from recombinant RAP30 and RAP74, to the depleted HeLa nuclear extract resulted in restoration of Tat transactivation. Of importance, the exogenous RAP74 was rapidly phosphorylated in the presence of Tat. These results suggest that RAP74 phosphorylation is one important step, of several, in the Tat transactivation cascade.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) encodes a highly conserved transactivator, Tat, that is expressed early in the viral life cycle and is essential for viral replication (Cullen, 1993; Jones and Peterlin, 1994; Gaynor, 1995; Karn, 1999; Garber and Jones, 1999; Jeang *et al.,* 1999). Tat stimulates transcription from the viral promoter located within the long terminal repeat (LTR) of the viral genome (Ratnasabapathy *et al.,* 1990; Marciniak *et al.,* 1990; Laspia *et al.,* 1989; Feinberg *et al.,* 1991; Kao *et al.,* 1987; Majello *et al.,* 1998). The primary effect of Tat is to increase the processivity of RNA polymerase II (RNAP II) that otherwise would prematurely terminate after the synthesis of short transcripts (Ratnasabapathy *et al.,* 1990; Laspia *et al.,* 1989; Feinberg *et al.,* 1991; Kao *et al.,* 1987). Tat has been shown to functionally interact with numerous components of the transcription complex, including transcription factor IID (TFIID) TATA binding protein (TBP) (Kashanchi *et al.,* 1994; Bohan *et al.,* 1992), $TAF_{II}250$ (Weissman *et al.,* 1998), RNAP II (Mavankal *et al.,* 1996; Cujec *et al.,* 1997a; Garcia-Martinez *et al.,* 1997), the CDK7 subunit of TFIIH (Cujec *et al.,* 1997b; Parada and Roeder, 1996), and the CDK9/cyclin T1 complex P-TEFb (Fujinaga *et al.,* 1998; Mancebo *et al.,* 1997; Wei *et al.,* 1998; Zhou *et al.,* 1998; Zhu *et al.,* 1997; Majello *et al.,* 1999; Bieniasz *et al.,* 1999; Garber *et al.,* 1998). To trigger efficient RNA chain elongation, either *in vivo* or *in vitro,* Tat must bind to the transactivation response (TAR) RNA stem-loop structure, which forms from the initial portion of the HIV-1 transcript (Berkhout *et al.,* 1989; Ratnasabapathy *et al.,* 1990; Bieniasz *et al.,* 1998; Wimmer *et al.,* 1999; Fujinaga *et al.,* 1999). It has been proposed that Tat affects several sequential steps during the subsequent steps of transcription elongation. Tat may target TFIIH directly and stimulate low-level phosphorylation of the carboxy-terminal domain (CTD) at an early step in transcription. Subsequently, Tat apparently recruits the cyclin T1/CDK9 complex, P-TEFb, to facilitate RNAP II elongation through the TAR RNA element. P-TEFb induces hyperphosphorylation of the CTD, leading to a transition from nonprocessive to processive transcription elongation (Fujinaga *et al.,* 1998; Jones, 1997; Mancebo *et al.,* 1997; Wei *et al.,* 1998; Zhou *et al.,* 1998; Zhu *et al.,* 1997; Isel and Karn, 1999).

The RNAP II-associated proteins RAP30 (26 kDa) and RAP74 (56 kDa) are subunits of the transcription factor TFIIF (Aso *et al.,* 1992; Chang *et al.,* 1993; Finkelstein *et al.,* 1992; Orphanides *et al.,* 1996; Yonaha *et al.,* 1993). TFIIF can bind RNAP II directly and recruit the enzyme to a preformed TFIID/TFIIB complex, which in turn stimulates the rate of RNAP II elongation (Lei *et al.,* 1998; Orphanides *et al.,* 1996). RAP74 is phosphorylated *in vivo,*

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in accordance with the presence of several potential phosphorylation sites in its primary sequence (Aso *et al.,* 1992; Finkelstein *et al.,* 1992; Yonaha *et al.,* 1997; Orphanides *et al.,* 1996). Phosphorylation of RAP74 has been reported to increase transcription initiation and elongation (Yonaha *et al.,* 1997; Kitajima *et al.,* 1994).

Several lines of evidence indicate that RAP74 is phosphorylated by TFIID and that the $TAF_{II}250$ subunit of TFIID selectively phosphorylates RAP74 on serine residues (Dikstein *et al.,* 1996; O'Brien and Tjian, 1998; Yonaha *et al.,* 1997). The results of Dikstein *et al.* (1996) indicate that $TAF_{II}250$ contains N- and C-terminal kinase domains. $TAF_{II}250$-bearing mutations within the N-terminal kinase domain exhibit a significantly reduced ability to rescue ts13 cells that express a temperature-sensitive $TAF_{II}250$ (O'Brien and Tjian, 1998).

The results presented here demonstrate that TFIIF is important for both basal and Tat-activated HIV-1 transcription. Depletion of RAP74 from extracts obliterated HIV-1 basal transcription and Tat transactivation. Importantly, the addition of reconstituted TFIIF from recombinant RAP30 and RAP74 to the depleted extract resulted in restoration of Tat transactivation. Our results further suggest that RAP74 is phosphorylated by $TAF_{II}250$ in Tat-activated transcription. Phosphorylation of RAP74 correlates with an enhanced transcription activity.

RESULTS

Tat stimulates the formation of transcriptionally active preinitiation complexes on the biotinylated HIV-1 LTR templates. The following studies were initiated to identify proteins that were involved in HIV-1 Tat transcription that might be regulated by posttranslational modification such as phosphorylation. HIV-1 LTR promoter templates (nt −110 to +168) were labeled with biotin at the upstream end (nt −110) as described under Materials and Methods. Biotinylated templates were incubated with HeLa nuclear extract in the absence or in the presence of Tat as described previously (Kashanchi *et al.,* 1994). Preinitiation complexes were subsequently isolated using streptavidin-coated magnetic beads and *in vitro* transcription assays were performed. In the absence of Tat, a low level of basal HIV-1 transcription was observed (Fig. 1A, lane 1). The addition of increasing amounts of Tat protein to the preincubation mix significantly increased transcription from the HIV-1 promoter (Fig. 1A, lanes 2–5). Consistent with previous results, optimum Tat transactivation was observed when approximately 100 ng of Tat was added to the reaction (Fig. 1A, lane 4).

The specificity of the *in vitro* transcription system is demonstrated in Figs. 1B through 1D. Tat failed to activate transcription from either a HIV-1 TATA box mutant or a TAR mutant template (Figs. 1B and 1C). Furthermore, transactivation was not observed when a Tat transactivation mutant, Tat72 C22G, was added to the transcription assay (Fig. 1D). Tat transactivation was sensitive to the addition of DRB and required the Tat-associated kinase P-TEFb (data not shown). These results, consistent with previous observations, suggest that preinitiation complexes formed in the presence of Tat have the factors required for activated transcription elongation (Isel and Karn, 1999). Moreover, the effect of Tat on transcriptional elongation may be observed with relatively short transcripts (Cujec *et al.,* 1997b; Okamoto *et al.,* 1996; Isel and Karn, 1999).

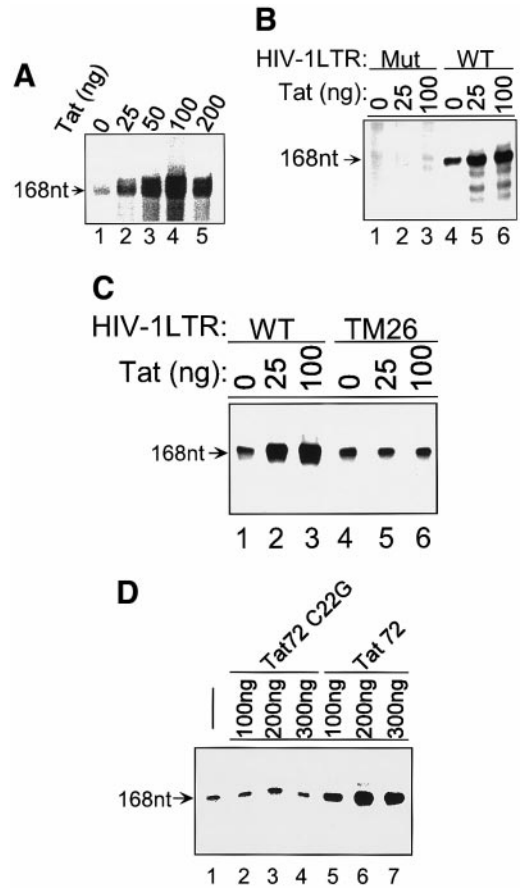

FIG. 1. Tat stimulated transcription from HIV-1 LTR. *In vitro* transcription reactions were performed with the purified preinitiation complexes and the transcripts were labeled with [α-^{32}P]UTP. (A) Tat stimulated transcription from the wild-type (WT) HIV-1 LTR. (B) No productive transcription from the TATA box mutant HIV-1 LTR (indicated as "Mut") was detected. (C) Tat was not able to activate transcription from the TAR mutant (TM26) HIV-1 LTR. (D) Comparison of the transactivation activities of wild-type GST–Tat72 and GST–Tat72 C22G proteins in the *in vitro* transcription assay.

RAP74 was specifically phosphorylated during Tat-activated transcription from the HIV-1 LTR. The ability to isolate preinitiation complexes allowed us to ask questions regarding the posttranslational modification of proteins during Tat transactivation. Specifically, the phosphorylation of the RNAP II CTD and other general transcription factors during Tat transactivation has been analyzed. Western blot analysis demonstrated that the

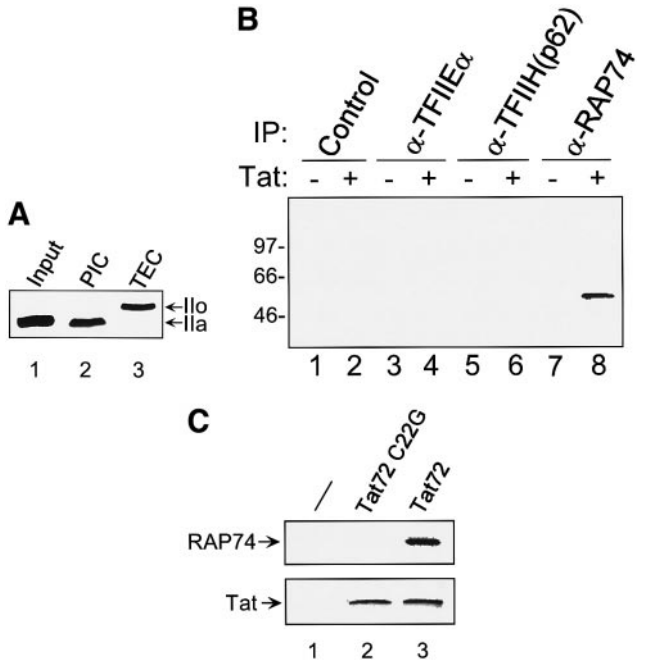

FIG. 2. Phosphorylation of RNAP II CTD and RAP74 in Tat-activated transcription. (A) Phosphorylation of RNAP II CTD in Tat transactivated transcription. Western blot analysis of Tat preinitiation complex (PIC) and Tat transcription elongation complex (TEC). Preinitiation complexes were assembled by incubating HeLa nuclear extract, biotinylated HIV-1 LTR templates, and poly(dI–dC) in the presence of Tat. Transcription reactions were initiated by the addition of nucleotides and allowed to incubate for 60 min at 30°C. To analyze the states of RNAP II CTD in the stages of transcription, the samples were fractionated on a 4% SDS polyacrylamide gel and then transblotted onto an Immobilon-P membrane (Millipore). RNAP II was detected with anti-RNAP II CTD antibody. (B) RAP74 phosphorylation in Tat-activated transcription. Transcription reactions were performed with the purified preinitiation complexes. The proteins phosphorylated during the transcription were labeled with [γ-^{32}P]ATP and immunoprecipitated with corresponding antibodies: lanes 1 and 2 with normal IgG (as controls), lanes 3 and 4 with anti-TFIIEα (p56) antibody, lanes 5 and 6 with anti-TFIIH (p62) antibody, lanes 7 and 8 with anti-RAP74 antibody; odd-numbered lanes without Tat and even-numbered lanes with Tat. (C) Comparison of the abilities of wild-type GST-Tat72 and GST-Tat72 C22G proteins to induce phosphorylation of RAP74 in the *in vitro* transcription assay. (Top) RAP74 phosphorylation; (bottom) Coomassie blue stain of the input Tat proteins.

primary species of RNAP II bound to the preinitiation complexes was unphosphorylated RNAP IIa (Fig. 2A, lanes 1 and 2). When nucleoside triphosphates were added to the reaction and transcription was allowed to proceed for 60 min, Western blot analysis of the elongation complexes demonstrated that RNAP II had been converted to the hyperphosphorylated RNAP IIo (Fig. 2A, lane 3), consistent with previous observations.

We next investigated whether general transcription factors were phosphorylated during Tat transactivation of the HIV-1 LTR. Preinitiation complexes were isolated and incubated with nucleoside triphosphates and [γ-^{32}P]ATP. Transcription reactions were then immunoprecipitated with control IgG or antibody specific for TFIIEα, the p62 subunit of TFIIH, or the RAP74 subunit of TFIIF. The results of this assay suggest that RAP74 was phosphorylated in transcription reactions containing the Tat protein (Fig. 2B, lanes 7 and 8). No phosphorylation of transcription factors such as TFIIEα and TFIIH p62 was found in the absence or in the presence of Tat (Fig. 2B, lanes 3, 4, 5, and 6). Western blot analysis of the immunoprecipitates demonstrated that comparable amounts of TFIIE, TFIIH p62, and RAP74 were precipitated from the transcription reactions (data not shown).

As a control for this experiment, we have analyzed the ability of the Tat72 C22G mutant to induce phosphorylation (Fig. 2C). The results presented in Fig. 2C demonstrate that RAP74 is phosphorylated in the presence of wild-type Tat72 (lane 3), but not the Tat transactivation-defective protein Tat72 C22G (lane 2). A Coomassie blue stain of the input Tat proteins demonstrates that an equal amount of protein was added to the reaction (Fig. 2C, bottom).

RAP74 was specifically phosphorylated by TAF$_{II}$250 in the Tat preinitiation complexes. It has been reported that the TAF$_{II}$250 kinase phosphorylates RAP74 (Dikstein *et al.,* 1996). To determine whether the TAF$_{II}$250 kinase was responsible for phosphorylation of RAP74 in the *in vitro* assays, HeLa nuclear extract was preincubated with anti-TAF$_{II}$250 for 60 min at 4°C. The anti-TAF$_{II}$250 antibody specifically blocks the kinase activity of TAF$_{II}$250, but does not affect incorporation of TFIID into the preinitiation complex (data not shown). In parallel assays, antibodies to CDK7 and CDK9 were added to the extract. Biotinylated HIV-1 LTR templates and Tat protein were then added to the extract. After incubation for 30 min at 30°C, the preinitiation complexes were purified with streptavidin-coated magnetic beads. [γ-^{32}P]ATP was added to the purified preinitiation complexes and the reaction was carried out for 30 min at 30°C. RAP74 was subsequently immunoprecipitated with anti-RAP74 antibody. RAP74 was not phosphorylated when the extract had been pretreated with TAF$_{II}$250 antibody (Fig. 3, lane 2). In contrast, phosphorylated RAP74 was observed when the extracts were pretreated with either anti-CDK7 or anti-CDK9 antibody (Fig. 3, lanes 1, 3, and 4). These results suggest that the TAF$_{II}$250 kinase specifically phosphorylates RAP74 in the Tat preinitiation complex.

Depletion of RAP74 from nuclear extracts significantly decreases HIV-1 transcription. To investigate the functional role of RAP74 phosphorylation in Tat transactivation, RAP74-depleted HeLa nuclear extract was prepared. Western blot analysis of the extracts before and after depletion demonstrate that RAP74 was removed from the extracts treated with anti-RAP74, but not control antibody (Fig. 4A). Subsequently, the extracts were used for *in vitro* transcription assays with HIV-1 LTR templates in the absence or in the presence of Tat. Removal of the endogenous RAP74 from HeLa nuclear extract obliter-

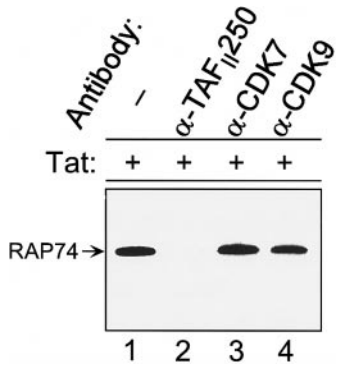

FIG. 3. RAP74 phosphorylation by TAF$_{II}$250 in Tat preinitiation complexes. HeLa nuclear extract (15 μl) was incubated for 60 min at 4°C with 3 μl of anti-TAF$_{II}$250, anti-CDK7, or anti-CDK9 antibody, respectively (normal IgG as a control). Biotinylated HIV-1 LTR templates (1.0 μg) and Tat protein (100 ng) were then added to pretreated extracts and the mixtures were allowed to incubate for 30 min at 30°C. Preinitiation complexes were separated with streptavidin-coated magnetic beads. Kinase reactions were performed with the purified preinitiation complexes and the phosphorylated proteins were labeled with [γ-^{32}P]ATP. Phosphorylated RAP74 was immunoprecipitated with anti-RAP74 antibody.

ated both basal and Tat-activated transcription from the HIV-1 LTR (Fig. 4B, lanes 2 and 7).

To demonstrate that the loss of transcription activity was due to depletion of RAP74, and not another general transcription factor, RAP30 and RAP74 were overexpressed and purified from bacteria (Imhof *et al.,* 1997). Subsequently, the RAP30/RAP74 TFIIF complex was reconstituted. Addition of purified, unphosphorylated RAP30/RAP74 to the RAP74-depleted HeLa nuclear extract restored basal transcription and Tat transactivation (Fig. 4B, lanes 4 and 9). Importantly, the results presented in Fig. 4C demonstrate that the exogenous RAP74 was rapidly phosphorylated in the reconstituted reactions containing Tat (Fig. 4C, lane 4), which exhibited a high level of transcription (Fig. 4B, lane 9). In contrast, reactions that did not contain Tat failed to phosphorylate the exogenous RAP30/RAP74 complex (Fig. 4C, lane 2) and exhibited a low level of transcription (Fig. 4B, lane 4). These results demonstrate that RAP74 is important for basal and Tat-activated transcription from the HIV-1 LTR. In the presence of Tat, phosphorylation of RAP74 is

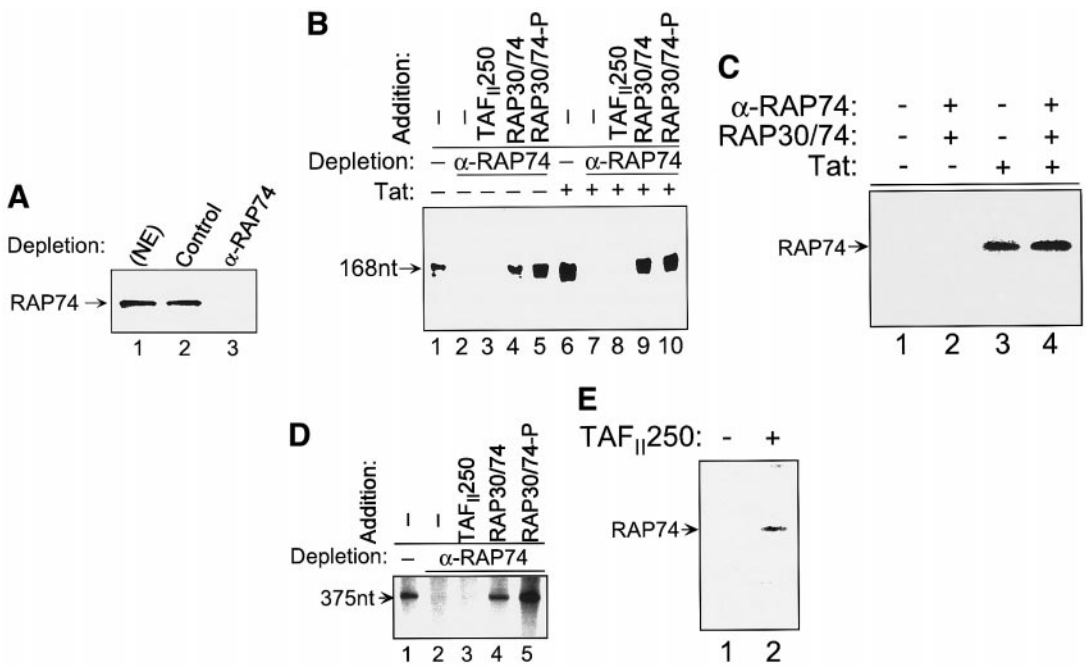

FIG. 4. Phosphorylated RAP74 enhanced the transcription activities of HIV-1 LTR and AdML. (A) Western blot analysis of RAP74 in mock-depleted HeLa nuclear extract and RAP74-depleted HeLa nuclear extract (NE, HeLa nuclear extract). (B) Phosphorylated RAP74 enhanced transcription from HIV-1 LTR. Transcription reactions were performed with mock-depleted HeLa nuclear extract (lanes 1 and 6) or RAP74-depleted HeLa nuclear extract (lanes 2–5 and 7–10) in the absence (−) or in the presence (+) of Tat. RAP30/RAP74 (unphosphorylated RAP74) or RAP30/RAP74-P (phosphorylated RAP74) was added to RAP74-depleted reactions as indicated in the figure (TAF$_{II}$250 was added as controls in lanes 3 and 8). (C) The exogenous RAP74 was phosphorylated during transcription in the presence of Tat. Transcription reactions were performed with RAP74-depleted HeLa nuclear extract complemented with RAP30/RAP74 or mock-depleted HeLa nuclear extract. RAP74 phosphorylated during transcription from HIV-1 LTR was labeled with [γ-^{32}P]ATP and immunoprecipitated with anti-RAP74 antibody. RAP74-depleted HeLa nuclear extract was complemented with RAP30/RAP74 in the presence of HIV-1 LTR templates without (lane 2) or with (lane 4) Tat. Mock-depleted HeLa nuclear extract was used as control in the presence of HIV-1 LTR templates without (lane 1) or with (lane 3) Tat. (D) Phosphorylated RAP74 stimulated the transcription activity of the adenovirus major late promoter. The transcription activity of adenovirus major late promoter was analyzed in extracts depleted with anti-RAP74 antibody and reconstituted with recombinant RAP30 and RAP74. (E) RAP74 phosphorylation by TAF$_{II}$250 in kinase assay.

observed, which correlates with a high level of transcription activity.

For comparison, similar assays were performed with the adenovirus major late promoter. Depletion of RAP74 from the extract abolished basal promoter activity (Fig. 4D, lanes 1 and 2). Addition of purified RAP74/RAP30 to the depleted extract restored transcription (Fig. 4D, lane 4). Addition of phosphorylated RAP74/RAP30 to the RAP74-depleted extract further stimulated transcription (Fig. 4D, lane 5). Thus, the basal AdML and HIV-1 promoters behaved similarly in that addition of phosphorylated RAP74/RAP30 increased transcription to an artificially high level, suggesting that phosphorylation of RAP74 is not an efficient process in the basal transcription complex.

Correlation between RAP74 phosphorylation and HIV-1 transcription activity. These results suggest that one limitation in HIV-1 transcription is the ability to phosphorylate the RAP74 subunit of TFIIF. To test this hypothesis, recombinant RAP74 was kinased with purified recombinant $TAF_{II}250$ (Imhof *et al.,* 1997) and subsequently reconstituted with RAP30. Addition of an equivalent amount of phosphorylated RAP30/RAP74-P (Fig. 4E) to the RAP74-depleted HeLa nuclear extract resulted in activation of basal transcription (Fig. 4B, lanes 4 and 5). Due to the ability of the Tat preinitiation complex to rapidly phosphorylate RAP74, addition of phosphorylated RAP30/RAP74-P did not increase Tat transactivation above the level seen with unmodified RAP74 (Fig. 4B, lanes 9 and 10). These studies demonstrate a strong correlation between RAP74 phosphorylation and HIV-1 transcription activity.

These results suggest that RAP74 is important for basal and Tat-activated transcription from the HIV-1 LTR. The basal HIV-1 transcription complex lacks the ability to induce $TAF_{II}250$ kinase activity on RAP74. In the presence of Tat, $TAF_{II}250$ kinase activity toward RAP74 is induced, providing an essential step in the cascade of events leading to the formation of an efficient transcription elongation complex. Moreover, it is likely that this step precedes the TAR-dependent Tat/P-TEFb hyperphosphorylation of the RNAP II CTD.

DISCUSSION

The results presented in this study demonstrate that phosphorylation of the RAP74 subunit of the transcription factor TFIIF is increased during Tat transactivation. Kitajima *et al.* (1994) have analyzed the regulation of TFIIF activity by phosphorylation by comparing the biochemical properties of alkaline phosphatase-treated TFIIF with those of native or bacterially expressed factors. Both the transcription initiation and the elongation stimulating activities of the phosphatase-treated TFIIF decreased to 15–20% of the native form under conditions in which the amount of TFIIF was rate-limiting. The decrease in transcription elongation was likely due to the fact that phosphatase-treated TFIIF bound to RNA polymerase less efficiently than native protein. In this regard, it is interesting to consider the results of Zawel *et al.* (1995), who demonstrated that TFIIF is unique in that it is the only basal transcription factor detected in the elongation complex. Following its release from the initiation complex, TFIIF has the ability to reassociate with RNAP II. Phosphorylation of RAP74 may facilitate the reassociation of the transcription factor with the elongation complex. In the event that the polymerase pauses, TFIIF may facilitate passage through the pause. It is interesting to speculate that phosphorylated TFIIF may help to "jumpstart" stalled or paused transcription complexes in which P-TEFb has phosphorylated the RNAP II CTD. In this model, phosphorylated TFIIF would not be absolutely required, but would facilitate the process of release of transcription elongation complexes. Phosphorylation of the RNAP II CTD by P-TEFb, on the other hand, is an absolute requirement for the paused complex to be released.

The 517-amino-acid (aa) sequence of RAP74 can be divided into three regions: (i) a highly basic N-terminal domain with significant globular structure (aa 1 to 217); (ii) an overall acidic, highly charged central region lacking in hydrophobic amino acids but rich in polar amino acids (aa 218 to 398); and (iii) a very basic C-terminal domain with globular structure (aa 399 to 517) (Aso *et al.,* 1992; Finkelstein *et al.,* 1992; Lei *et al.,* 1998). The N-terminal domain is important for RAP30 binding (Wang and Burton, 1995; Yonaha *et al.,* 1993), preinitiation complex assembly, and elongation (Lei *et al.,* 1998). The C-terminal domain makes contact with TFIIB and RNAP II and stimulates the activity of a CTD phosphatase (Chambers *et al.,* 1995). Of significance, sequences within the central region affect accessibility of the C-terminal domain with RNAP II (Wang and Burton, 1995). The evidence that serine residues within amino acids 217–224, located in the central region of RAP74, are phosphorylated by $TAF_{II}250$ (Dikstein *et al.,* 1996; Yonaha *et al.,* 1997) suggests the possibility that the functional role of RAP74 phosphorylation by $TAF_{II}250$ may be related to increasing the interaction with RNAP II. This interpretation would be consistent with the results of Kitajima *et al.* (1994), who reported that alkaline phosphatase treatment of RAP74 reduced the interaction with RNAP II.

The general TFIID, which is composed of TBP and multiple TBP-associated factors (TAFs) (Goodrich and Tjian, 1994; Orphanides *et al.,* 1996), is one of the central components involved in regulation of RNAP II transcription (Orphanides *et al.,* 1996; Sauer *et al.,* 1995; Verrijzer and Tjian, 1996). The largest component of the TFIID protein complex, $TAF_{II}250$, provides the core scaffold upon which TBP and the other TAFs are assembled, forming a stable holo-TFIID complex (Weinzierl *et al.,* 1993). Of interest, $TAF_{II}250$ is identical to the cell cycle

gene 1 (CCG1), a gene able to complement the late G1 arrest observed in the temperature-sensitive mutant hamster cell line ts13 (Hisatake *et al.,* 1993; Ruppert *et al.,* 1993). There are multiple domains within the $TAF_{II}250$ protein that appear to carry out potentially different functions. First, several lines of evidence suggest that $TAF_{II}250$ is a major scaffold protein of TFIID and is thus involved in both core–promoter recognition and coactivator function through protein–protein interactions (Verrijzer *et al.,* 1995; Verrijzer and Tjian, 1996; Wang and Tjian, 1994; Wang *et al.,* 1997). Second, both human $TAF_{II}250$ and *Drosophila* $TAF_{II}250$ contain a histone acetyltransferase domain that can acetylate histones H3, H4, and H2A (Mizzen *et al.,* 1996). Finally, $hTAF_{II}250$ contains two independent serine/threonine kinase domains, located in the N- and C-terminal regions of the protein (Dikstein *et al.,* 1996). Neither of these domains resembles other known kinases, suggesting that they may represent members of a novel class of kinases. Both the N- and the C-terminal kinase domains of $TAF_{II}250$ can phosphorylate RAP74, but efficient phosphorylation of RAP74 requires the combined action of both domains. It is interesting to consider that the kinase activity of $TAF_{II}250$ may be induced by an activator(s). Along these lines, Weissman *et al.* (1998) have reported that Tat interacts with $TAF_{II}250$ and regulates acetyl transferase enzyme activity. Preliminary evidence suggests that Tat increases the kinase activity of purified $TAF_{II}250$ on RAP74 (data not shown). Experiments are under way to examine the effect of Tat on $TAF_{II}250$ kinase activity in the TFIID complex.

Kato *et al.* (1992) have previously analyzed the importance of TFIIF in HIV-1 basal and Tat-activated transcription. Preincubation of nuclear extracts with an antibody raised against recombinant RAP74 decreased both basal and Tat-activated transcription. The results reported in our present study extend these findings by demonstrating that phosphorylation of the RAP74 subunit of TFIIF occurs only in the presence of Tat and correlates with increased transcriptional activity. $TAF_{II}250$ has been reported to phosphorylate RAP74 at serine residues in amino acids 217–224. It will be of interest to develop mutations within the phosphorylation domain of RAP74 (amino acids 217–224) (Dikstein *et al.,* 1996; Yonaha *et al.,* 1997) and analyze their effect on basal and Tat-activated transcription. Moreover, these mutations would be important in determining the effect of phosphorylation of TFIIF on transcription activity using other promoters.

It is becoming increasingly apparent that RAP74 phosphorylation appears to increase TFIIF transcription activity. Kitajima *et al.* (1994) have shown that RAP74, but not RAP30, was extensively phosphorylated *in vivo* and that TFIIF transcription initiation and elongation activities were stimulated by phosphorylation of RAP74. Consistent with these observations, Yonaha *et al.* (1997) have shown that the cell-cycle-dependent phosphorylation of RAP74 increases *in vitro* transcription activity. In view of the fact that the RAP74 binding site of $TAF_{II}250$ is required to rescue ts13 cells, the cell-cycle-dependent phosphorylation of RAP74 may play an important role in cell cycle regulation. The viral transactivator Tat may accentuate this function of $TAF_{II}250$ and stimulate HIV-1 transcription. Given that mutations in TBP that affect Tat transactivation correlate with the ability of TBP to bind $TAF_{II}250$, it is likely that the multisubunit TFIID complex, and not TBP, is involved in HIV-1 transcription (Pendergrast *et al.,* 1996). In view of the results presented in this article, the kinase activity $TAF_{II}250$ may also play a significant role in the efficiency of Tat transactivation.

MATERIALS AND METHODS

Biotinylation of template DNAs. The wild-type, TATA box mutant, and TAR mutant TM26 (Boris-Lawrie *et al.,* 1992) HIV-1 LTR templates (nt −110 to +168) were amplified by PCR with the forward primer 5′ biotinylated-TAT GGA TTT ACA AGG GAC TTT C-3′ and the reverse primer 5′-GAT CCG ATT ACT AAA AGG G-3′. The primers were synthesized and biotinylated by Lofstrand Labs Limited.

Isolation of preinitiation complexes. Assembly reactions (50 μl) contained 25 μl of HeLa nuclear extract, 1.0 μg of biotinylated HIV-1 LTR template, 1.0 μg of poly(dI–dC), purified Tat protein in 1× *in vitro* transcription (IVT) buffer [IVT buffer: 50 mM KCl, 6.25 mM $MgCl_2$, 20 mM HEPES (pH 7.9), 2 mM DTT, 0.5 mM EDTA (pH 8.0), 10 μM $ZnSO_4$, 10 mM creatine phosphate, 100 μg/ml of creatine kinase, and 8.5% glycerol]. After a 30-min incubation at 30°C, streptavidin-coated magnetic beads (Dynabeads, Dynal) preequilibrated in binding buffer [20 mM HEPES (pH 7.9), 80 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 10 μM $ZnSO_4$, 100 μg/ml bovine serum albumin (BSA), 0.05% Nonidet-P40 (NP-40), and 10% glycerol] were added to the reaction mixtures. The reaction mixes were then incubated for an additional 30 min at 30°C. The immobilized templates were harvested using a magnetic stand and the preinitiation complexes were washed extensively with 1× IVT buffer. IVT and Western blot analysis were performed using the purified preinitiation complexes assembled on the immobilized templates.

In vitro transcription with the purified preinitiation complexes. In vitro transcription reactions (100 μl) were set up by resuspending the purified preinitiation complexes in 100 μl of 1× IVT buffer, 50 μM ATP, 50 μM CTP, 50 μM GTP, 10 μCi [α-^{32}P]UTP, and 10 units of RNasin (Promega). The transcription reaction mixtures were incubated for 60 min at 30°C. RNA transcripts were purified and fractionated by electrophoresis on a 6% denaturing polyacrylamide gel containing 7 M urea in 1× TBE. The labeled mRNA products were detected with a PhosphorImager.

Western blot analysis of the purified preinitiation complexes. The purified preinitiation complexes, assembled on the immobilized templates, were heated in SDS loading buffer for 10 min at 100°C. The released proteins were fractionated by electrophoresis on 4% SDS–polyacrylamide gels and then transblotted onto 0.45 μM Immobilon-P membranes (Millipore). RNAP II was detected with a specific antibody against the RNAP II CTD (Santa Cruz Biotechnology).

Immunoprecipitation. To detect phosphorylated proteins during transcription, reaction mixtures (100 μl) containing the purified preinitiation complexes, 50 μl of 2× IVT buffer, 50 μM CTP, 50 μM GTP, 50 μM UTP, 10 μCi[γ-^{32}P]ATP, and 10 units of RNasin (Promega) were incubated for 60 min at 30°C. Four hundred microliters of IP buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA (pH 8.0), 5% glycerol] was added to the reaction mixtures and the streptavidin-coated beads were separated using a magnetic stand. Six microliters of anti-RAP74, anti-TFIIEα, and anti-TFIIH(p62) antibodies was added to the supernatants and the mixtures were gently rocked overnight at 4°C. Protein A/Sepharose CL-4B beads were added to the mixtures containing antigen/antibody complexes and the mixtures were incubated for 60 min at 4°C with rocking. The protein A/antibody/antigen complexes were harvested and washed four times with RIPA buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS]. The samples were analyzed by 4–20% SDS–polyacrylamide gel electrophoresis and PhosphorImager.

Assay of RAP74 phosphorylation in purified preinitiation complexes. Fifteen microliters of HeLa nuclear extract and 3 μl of anti-TAF$_{II}$250, anti-CDK7, or anti-CDK9 antibodies were mixed and incubated for 60 min at 4°C. Preinitiation complexes were assembled by adding 1.0 μg biotinylated HIV-1 promoter template and 100 ng Tat protein to the mixtures. After a 30-min incubation at 30°C, the preinitiation complexes were isolated with streptavidin-coated magnetic beads. Kinase reactions were performed by mixing the purified preinitiation complexes with 10 μCi [γ-^{32}P]ATP in a total volume of 100 μl. After incubation for 30 min at 30°C, the preinitiation complexes were purified using the magnetic stand. Five hundred microliters of IP buffer was added to the tubes and the mixtures were incubated for 120 min at 4°C with rocking. The supernatants were collected and anti-RAP74 antibody (Santa Cruz Biotechnology) was added to the supernatants to immunoprecipitate phosphorylated RAP74.

TAF$_{II}$250 kinase assay. Kinase assays were performed in 30-μl reaction mixtures containing 40 ng RAP74, 20 ng TAF$_{II}$250, 10 μCi [γ-^{32}P]ATP, and 1× kinase buffer [50 mM Tris–HCl (pH 7.5), 5 mM DTT, 5 mM $MnCl_2$, 4 mM $MgCl_2$, 40 mM KCl, 0.25 mg/ml BSA, 2% glycerol, and 0.1% NP-40]. The reactions were incubated for 30 min at 30°C and analyzed on 4–20% SDS–polyacrylamide gel.

Immunodepletion of RAP74 from HeLa nuclear extract. HeLa nuclear extract (50 μl) in 0.8 M KCl buffer D [20 mM HEPES (pH 7.9), 15% glycerol, 800 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA (pH 8.0), 0.1% NP-40, and 1 mM DTT] was incubated with 20 μl of Protein A–Sepharose beads to which anti-RAP74 antibodies had been prebound (10 μg of IgG, Santa Cruz Biotechnology). Antibody-bound complexes were removed by centrifugation. After the procedure was repeated twice, depleted nuclear extract was dialyzed against 0.1 M KCl buffer D and assayed by Western blot analysis.

Transcription activity assay of phosphorylated RAP74. RAP74 was phosphorylated using purified TAF$_{II}$250 *in vitro,* as above. RAP30/RAP74-P (phosphorylated) complexes were formed by mixing purified, phosphorylated RAP74 with purified RAP30. Transcription reactions were performed using RAP74-depleted HeLa nuclear extract complemented with RAP30/RAP74 or RAP30/RAP74-P.

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